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Detection of anthelmintic resistance in goats of an organized farm of Assam through *in vitro* egg hatch assay

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Abstract
A study was undertaken to determine resistance against albendazole (ALB), fenbendazole (FBZ) and closantel (CLS) in gastrointestinal nematodes of goat in an organized farm of Assam. The resistance was first investigated using faecal egg count reduction test (FECRT) in naturally infected goat which was further confirmed by *in vitro* egg hatch assay (EHA). Pre- and post-treatment copro-culture was performed to identify the species and genera of nematodes. In an organized goat farm of Assam the anthelmintics like albendazole, fenbendazole and closantel were found to be resistant i.e. the percent efficacy was not observed more than 95% of these three anthelmintics. The ED_{50} value for fenbendazole, albendazole and closantel was recorded as 0.3506 µg/ml, 0.3357 µg/ml and 0.2330 µg/ml, respectively. The relative potency of closantel with respect to fenbendazole was found to be 66.46 per cent indicates small amount of closantel is sufficient to 0.3506 amount of fenbendazole. *In vitro* egg hatch assay after logistic fit of response using fenbendazole, albendazole and closantel were estimated. The results of the survey indicated multiple resistance in *Haemonchus contortus* and *Trichostrongylus* spp. to benzimidazoles and closantel in this farm.

Keywords: Anthelmintic resistance, goat, egg hatch assay, albendazole, fenbendazole, closantel, Assam

Introduction
Gastrointestinal nematodes, adversely affect animal production causing huge economic losses all over the world [1] and the livestock in India is no exception to it. Use of chemicals for the treatment and control of gastrointestinal parasites is most widely practiced throughout the world [2]. Development of AR to commercially available drugs has, however, become a serious problem [3, 1]. In many parts of the world, therefore, anthelmintics are loosing their efficacy especially in goats, e.g. in Europe [4], Africa [5], Australia [6], New Zealand [7] and from India in sheep and goat [8, 9, 10].
In India, one of the important factors of high prevalence of nematodes in goats [11] may be the treatment failure with the commonly used anthelmintics. The present study was, therefore, carried out to screen the gastrointestinal nematodes of goats for the development of resistance against albendazole (ALB), fenbendazole (FBZ) and closantel (CLS) in an organized farm of Assam.

Materials and Methods
The study was conducted at Goat Research Station (GRS), Burnihat under Assam Agricultural University. For this study egg hatch assay (EHA) and faecal egg count reduction test (FECRT) was conducted on faecal samples collected from animals of GRS, Burnihat. The animals were maintained under semi-intensive system of management. The flock of Assam local and crossbred goats were let loose for grazing from 9 am to 4.30 pm, while the Beetal goats were mainly stall-fed and allowed to graze only from 3.00 to 4.30 pm. The animals were daily supplied with 200 g of concentrate mixture along with 3-4 kg of chaffed fodder. The animals were housed in permanent shed with asbestos roof and a raised floor made of wooden planks. The side walls were consists two parts: half of the wall was of wooden planks over which stood wire netting. Regular deworming was carried out under a schedule programme *viz*., once in three months. The previous history of drugs used for deworming for the last 10 years were carried out by albendazole, fenbendazole, closantel and ivermectin. During last five years, it was observed that scheduled anthelmintic drenching programme did not produce effective control of nematodes. Hence, it was necessary to evaluate the efficacy of anthelmintics in this farm. Therefore, faecal egg count reduction test (FECRT) and *in vitro* egg hatch assay (EHA) were performed to confirm any resistance problem in the flock.

Faecal egg count reduction test (FECRT)

A total of 112 (44 local goats, 24 crossbred and 44 Beetal goats) naturally infected goats (6 months to 3 ½ years old) showing strongyle type egg counts of more than 500 per gram of faeces were randomly divided into 4 groups. Goats of group A, B and C were orally drenched with fenbendazole (FBZ), albendazole (ALB) and closantel (CLS) at the rate of 7, 5 and 10 mg/kg body weight, respectively. Group IV was kept as untreated control.

Faecal samples were collected from the rectum of each goat on day 0 and 14 post-drenching. Faecal egg counts (EPG) were determined by stoll's technique [12]. Samples were pooled for each group and cultured for isolation and identification of nematode larvae for 10 days [13].

***In vitro* egg hatch assay (EHA)**

Faecal samples were collected per rectum from the groups of goat which were to be treated with Benzimidazole (albendazole and fenbendazole) and closantel. The eggs were recovered using flotation technique [14]. EHA was then performed as described by Coles [14]. The concentration of fenbendazole (FBZ), albendazole (ALB) and closantel (CLS) were used ranged from 0.0625 to 8.0 µg/ml.

Estimation of anthelmintic efficacy

Percent faecal egg count reduction and efficacy for each anthelmintic were calculated on RESO compute programme [14]. ED_{50} value was calculated for the eggs by Reed-Muench method as detailed by Charles [15]. Logistic fit available in SAS 9.3 was used to estimate ED_{50} and test is favour of hatched egg at 95% confidence limit.

Results and Discussion

Based on FECRT, it was found that FBZ, ALB and CLS had an efficacy less than 95% against gastrointestinal nematodes in case of local, crossbred and Beetal goats in Goat Research Station, AAU, Burnihat (Table 1). These data indicated the presence of anthelmintic resistance against G.I. nematodes in this farm, based on Coles [14] who reported that there was resistance when an anthelmintic showed efficacy less than 95% and the lower 95% confidence limit was less than 90%.

Strongyle and strongyloides spp. larvae were identified on pre-drench faecal culture and during post-drench faecal culture. *Haemonchus contortus* was found only predominant general throughout the study period.

Results of the post-treatment faecal culture of the present study suggested that resistance was predominantly against *Haemonchus contortus*. This was in agreement with the finding of Van Wyk [16] who suggested that resistance often arises quickly in *Haemonchus contortus* than in other nematodes. On the other hand, *Haemonchus contortus* larvae on post-drenching culture were probably from those worms which survived the treatment.

Table 1: Mean±SE faecal egg count and percent efficacy for FBZ, ALB and CLS in goats infected with gastrointestinal nematodiosis

Breed	Group	EPG (Mean±SE)	% Efficiency	95% confident limit		Results
				Lower	Upper	
Local	Control	1836.36±199.67	-	-	-	-
	Fenbendazole	490.91±65.30	73.27	61.99	81.20	Resistant
	Albendazole	400.00±42.64	78.22	69.00	84.68	Resistant
	Closantel	200.00+35.68	89.11	84.52	92.34	Resistant
Crossbred	Control	1800.00±315.17	-	-	-	-
	Fenbendazole	383.33±79.23	78.70	67.92	85.86	Resistant
	Albendazole	416.67±83.33	76.85	65.13	84.63	Resistant
	Closantel	366.67±88.19	79.63	69.31	86.48	Resistant
Beetal	Control	2145.45±357.10	-	-	-	-
	Fenbendazole	845.45±114.70	60.59	38.83	74.61	Resistant
	Albendazole	818.18±163.38	61.86	40.80	75.43	Resistant
	Closantel	518.18±72.39	75.85	62.51	84.44	Resistant

The results of the *in vitro* egg hatch assay for benzimidazole (fenbendazole and albendazole) and closantel are shown in Table 2. The ED_{50} value for fenbendazole, albendazole and closantel was recorded as 0.3506 µg/ml, 0.3357 µg/ml and 0.2330 µg/ml, respectively in Goat Research Station (GRS), Burnihat.

Table 2: Results of logistic fit of response, estimates of parameters and ED_{50} values of albendazole, fenbendazole and closantel drugs for hatched larvae

Drug (µg/ml)	Intercept (a)	Concentration (b)	Chi Sq (X^2)	P value	ED_{50}	95% confidence limit	
						Lower	Upper
Albendazole	2.48±0.24	-7.38±0.77	91.8	<0.0001**	0.3357	0.3016	0.3764
Closantel	1.85±0.21	-7.95±0.86	84.97	<0.0001**	0.2330	0.2038	0.2650
Fenbendazole	2.72±0.25	-7.77±0.79	97.06	<0.0001**	0.3506	0.3170	0.3907

**Significant at P<0.0001

Since probit and logit (Fig. 1, 2 and 3) model are statistically at par for finding ED_{50} (medium effective dose), logit model for each treatment group was presented in Table 2. The constant of linear regression coefficient in case of all treated group was positive while slope parameter in each treated group was negatively showing a decrease in respect in percentage. The significantly X^2 test depicts the model may not be adequate due to possibly non inclusion of relevant concomitant variables in the study. As a study from theoretical perspective the ED_{50} with 95% confidence limit was displayed in the Table 2. It appears that the highest ED_{50} was observed in fenbendazole (0.3506) with lowest in closantel (0.2330). The relative potency of closantel with respect of fenbendazole was found to be 66.46 per cent indicates small amount of closantel is sufficient to 0.3506 amount of fenbendazole.

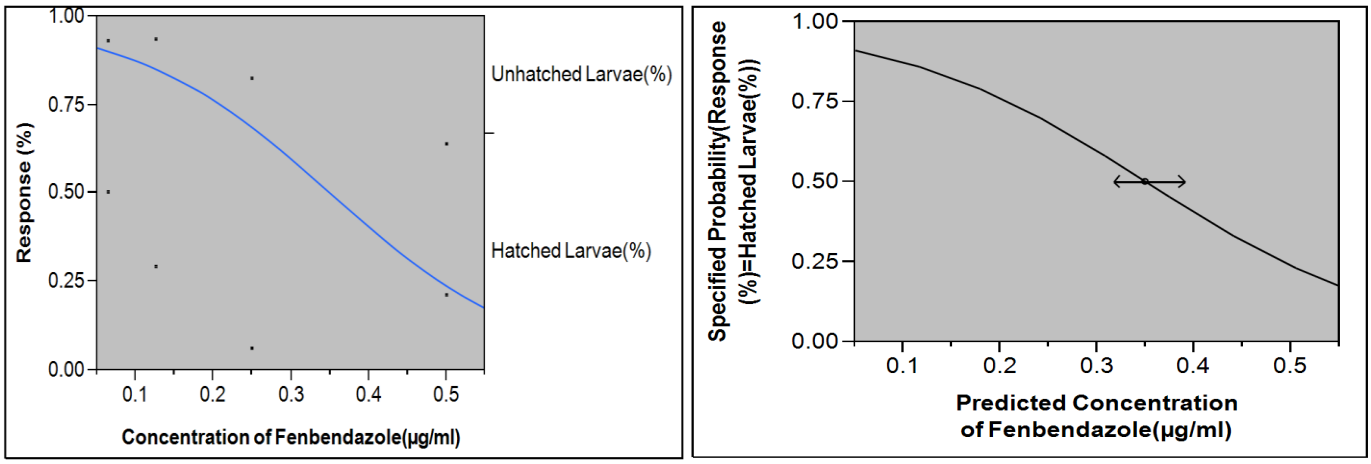

Fig 1: Showing *in vitro* egg hatch assay after logistic fit of response using fenbendazole

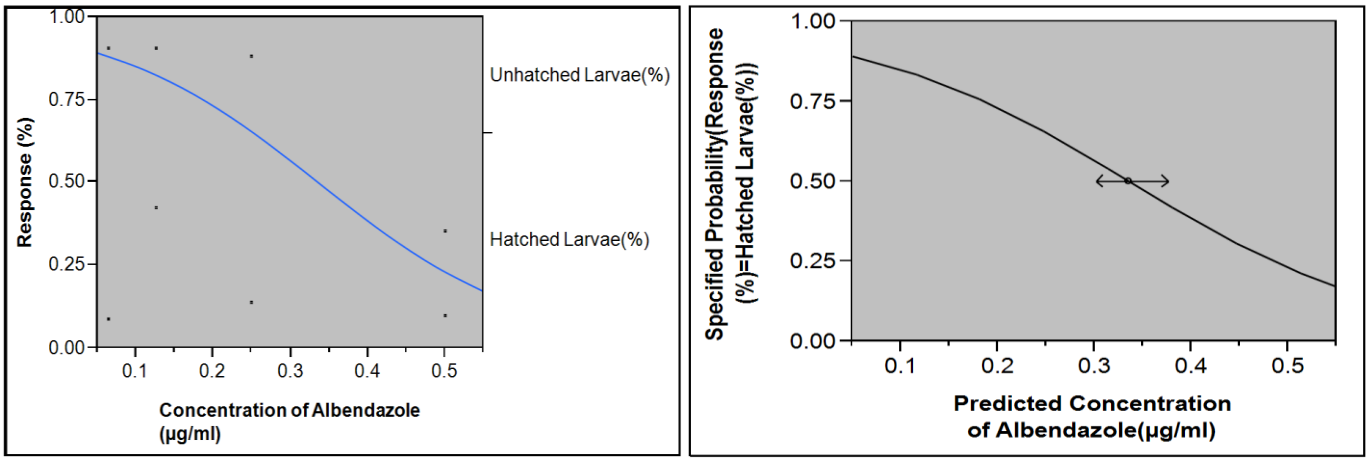

Fig 2: Showing *in vitro* egg hatch assay after logistic fit of response using albendazole

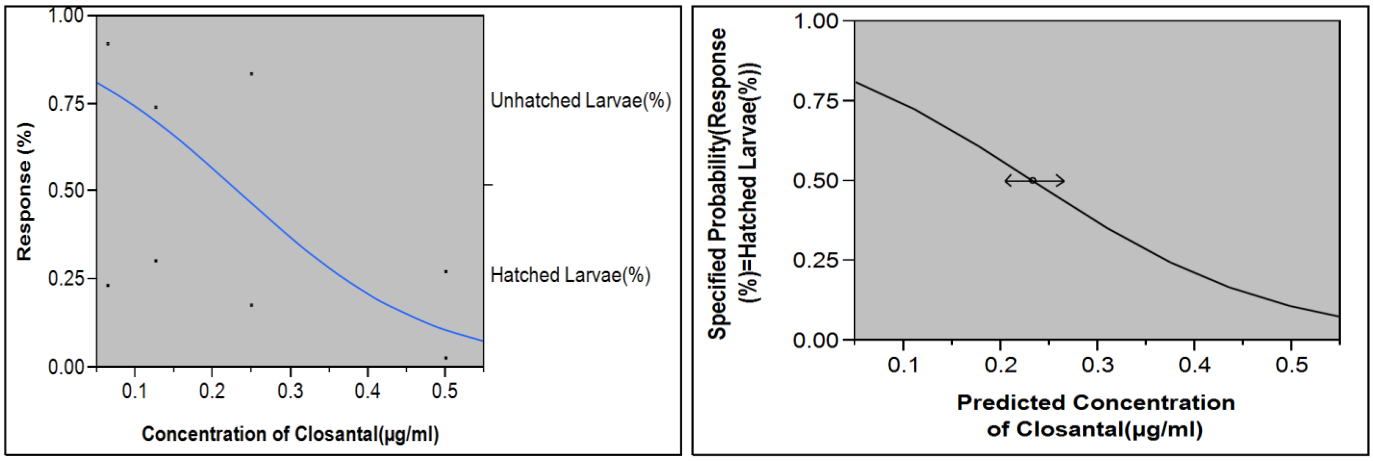

Fig 3: Showing *in vitro* egg hatch assay after logistic fit of response using closantel

According to Coles [14], eggs with an ED_{50} value in excess of 0.1 µg thiabendazole /ml is indicator of benzimidazole resistance. The ED_{50} value of 0.3506 µg fenbendazole/ml and 0.3357 µg albendazole/ml obtained in the egg hatch assay for benzimidazole group was confirmed as resistant, as it was well above the limit prescribed by Coles [14].

Similar findings of benzimidazole resistance with ED_{50} value greater than 0.10 µg/ml of thiabendazole was previously recorded by [17] from India. Recently, Dinesh [18] observed *in vitro* egg hatch assay for albendazole which revealed ED_{50} was 0.196 with lower and upper limit of ED_{50} of 0.051 and 0.329, respectively, indicating resistance to bendimidazole.

The ED50 value obtained after logistic fit of response for closantel as a reference in the egg hatch assay was 0.2330 µg/ml and its 95% confidence limit upper and lower was 0.2650 and 0.2038, respectively (Table 2 and Fig. 3).

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